Pestic. Sci. 1998, **53**, 91–95

[^{14}C]Dicofol Association to Cellular Components of *Azospirillum lipoferum*

Denise Maria Soares Mano & Tomaz Langenbach*

Instituto de Microbiologia, Universidade Federal do Rio de Janeiro, C.P. 68-040, CEP: 21941-590, Rio de Janeiro, RJ, Brazil

(Received 8 September 1997; accepted 10 December 1997)

Abstract: The bacterium *Azospirillum lipoferum* is able to survive in high concentrations of the organochlorine acaricide dicofol [1,1-bis-(4-chlorophenyl)-2,2,2-trichloroethanol]. It accumulates this chemical in the cell envelope where it is protected against hydrolysis. We investigated the nature of cell envelope molecules with which [^{14}C]dicofol is associated; no indication of [^{14}C]dicofol–saccharide bonds was found. We concluded that about 80% of the total [^{14}C]dicofol found in the cells was associated with lipids and the remaining 20% with proteins. Electrophoresis did not indicate any correlation of a specific protein band with [^{14}C]dicofol radioactivity peaks. After Folch partition, [^{14}C]dicofol distribution in TLC analysis showed 60% of [^{14}C]dicofol–lipid bonds related to neutral lipids, 20% to phospholipids and the remaining 20% of the bonds associated with other lipids. Experimental results suggested that [^{14}C]dicofol associates mainly with membrane domains near proteins and that this association influences membrane fluidity as well as enzymatic activity.

Pestic. Sci., **53**, 91–95 (1998)

Key words: dicofol; *Azospirillum*; insecticide microbiology; organochlorine adsorption

1 INTRODUCTION

Azospirillum lipoferum (Beijerinck) Tarrand, Kreig & Dobereiner, chosen as a model, is a Gram-negative nitrogen-fixing bacterium ubiquitously distributed in soil and able to survive in high concentrations (0·135 to 0·270 mM; 50 to 100 mg litre^{-1}) of the organochlorine acaricide dicofol [1,1-bis-(4-chlorophenyl)-2,2,2-trichloro-ethanol]. As part of its response, this bacterium develops cyst-like structures after 48 to 72 h in a medium containing dicofol.[1] *A. lipoferum* survival also involves others escape mechanisms, possibly including its capacity to biodegrade this substance. It is well known that dicofol is easily degraded in abiotic conditions due to hydrolytic reactions.[2] Data obtained in our laboratory showed that *A. lipoferum* does not biodegrade dicofol, and that 69% of the pesticide molecule is preserved in the presence of *A. lipoferum*.[3] In control samples, without cells, only 33% of dicofol molecules are recovered. This micro-organism bioaccumulates dicofol (bioconcentration factor of 577), protecting this acaricide against hydrolysis.[3] We observed that up to 90% of this acaricide remains in the *A. lipoferum* cell envelope and only about 10% is found in the cytosol.[3] The purpose of the present work was to identify the nature of the cell molecules with which [^{14}C]dicofol associates. This approach may allow an understanding of why dicofol is preserved from hydrolysis when it is bioaccumulated, and give some insights into the cellular mechanism of action of organochlorine chemicals in unicellular organisms.

As organochlorine–cell associations are weak, we used the photo-induced binding technique.[4] This method overcomes the problem of pesticide loss and redistribution during the washing procedures. It allows the localisation of [^{14}C]dicofol in the cell because it stabilises reversible non-covalent bonds of small ligands to biological macromolecules. The new covalent bonds are

* To whom correspondence should be addressed.

 Pestic. Sci. 0031-613X/98/$17.50. Printed in Great Britain

resistant to washing and fractionation procedures. The [^{14}C]dicofol—a small ligand—must be photoactivable after a brief exposure to an ultraviolet radiation of high intensity. Reactive intermediates of the photolysed ligand bind instantaneously to neighbouring cell molecules. We investigated the [^{14}C]dicofol association with cell-surface saccharides, proteins and lipids, as they constitute the main components of the bacterial membrane.

2 EXPERIMENTAL METHODS

2.1 Chemicals

The dicofol standard (99%) was purchased from Riedel-de-Haen. The commercial dicofol 185 g litre^{-1} EC, Kelthane EC, and the [*U*-^{14}C]dicofol (UL), specific radioactivity 19·99 μCi g^{-1}, were kindly supplied by Rohm & Haas Company. All chemicals were P.A. grade.

2.2 Micro-organism

Azospirillum lipoferum SpBr 17 (ATCC 29709) was kindly supplied by Dra J. Dobereiner (Embrapa, km 47, Rio de Janeiro). It was maintained on semi-solid potato medium.[5]

2.3 Incubation conditions

Experiments were run in Erlenmeyer flasks containing nitrogen-free broth (NFb) supplemented with ammonium nitrate (0·8 g litre^{-1}; pH 6·8–7·0.[5] Cells were obtained from an overnight exponential growth in a rotatory bath at 32°C. The initial inoculum concentration was 5×10^5 cell ml^{-1}.

2.4 [^{14}C]Dicofol photo-induced binding to *Azospirillum lipoferum* cells

In order to determine the nature of the *A. lipoferum* molecules with which dicofol was associated, each Erlenmeyer flask containing NFb medium received a final concentration 3×10^9 cell ml^{-1} of exponential phase bacteria cells and 5 mg [^{14}C]dicofol litre^{-1} (0·0135 mM) obtained from the stock solution of [^{14}C]dicofol in methanol (1 g litre^{-1}) and directly added to the medium. It was then incubated for 30 min in a rotatory bath at 32°C preceding the photo-induced binding method. Cells were irradiated in 1·5 ml aliquots for 15 s in 6-cm diameter culture dishes placed 2·5 cm below a Philips HPK 125-W mercury lamp.[4] After irradiation, cells were washed twice with acetone + water (3 + 1 by volume) to remove any unbound [^{14}C]dicofol. Aliquots to measure the protein content in the cells were taken by the Bradford method[6] and the radioactivity by liquid scintillation counting in toluene + Triton X-100 + PPO + POPOP, (900 ml to 100 ml + 5·5 g + 0·1 g). Controls were run without UV irradiation.

2.5 [^{14}C]Dicofol association with *Azospirillum lipoferum* surface saccharides

To determine the amount of [^{14}C]dicofol associated with *A. lipoferum* surface saccharides, bacterial cells were pre-treated with sodium periodate (0·1 mM) for 5 min at 32°C in order to promote saccharide oxidation, and then washed with phosphate buffer. Cells were incubated with [^{14}C]dicofol and submitted to the photo-binding method. Protein content and [^{14}C]dicofol radioactivity were determined as described above. Controls were run using cells without pre-treatment with 0·1 mM sodium periodate.

Wheat germ agglutinin (WGA) and *Limulus polyphenus* agglutinin (LPA)—which are lectins specific for sialic acid—were used to test *A. lipoferum* agglutination capacity at normal conditions and to check the expected surface saccharide oxidation after treatment with 0·1 mM sodium periodate.

2.6 [^{14}C]Dicofol association with *Azospirillum lipoferum* proteins

After photo-induced binding treatment, the cell suspensions were centrifuged at 5000*g*, and the cell pellets were suspended in mercaptoethanol + sodium dodecyl sulphate buffer for 5 min at 100°C. Samples were then centrifuged for 5 min at 5000*g* and the supernatant was submitted to 15% polyacrylamide gel electrophoresis, PAGE.[7] The gel was photographed, dried and cut in 0·2-cm slices. After digestion overnight at 50°C in hydrogen peroxide (300 g litre^{-1}; 0·5 ml), bound radioactivity was estimated by liquid scintillation counting.

2.7 [^{14}C]Dicofol association with *Azospirillum lipoferum* lipids

After the photo-induced binding treatment, crude lipids were extracted from the cell pellet with chloroform + methanol (2 + 1 by volume) overnight and chloroform + methanol (1 + 2 by volume) for 1 h at room temperature in a shaker. The crude extracts were purified by Folch partition[8] with chloroform + methanol + 7·5 g litre^{-1} aqueous KCl (4 + 8 + 3 by volume). Samples were concentrated for TLC fractionation on silica plates with chloroform + methanol + water (65 + 35 + 7 by volume). Lipid spots were detected by iodine vapour.

TABLE 1
Azospirillum lipoferum Agglutination with Lectins in Sodium-Periodate-Treated and Untreated Cells

Lectins (*g litre*$^{-1}$)	*LPA*		*WGA*	
	Untreated	*Treated*	*Untreated*	*Treated*
250	+++	−	+++	−
125	+++	−	++	−
62·5	+++	−	++	−
31·3	++	−	+	−
15·6	++	−	−	−
7·81	+	−	−	−

The silica plate was scraped in 0·5-cm slices and the radioactivity of each slice was determined by liquid scintillation counting.

3 RESULTS

3.1 [^{14}C]Dicofol association with *Azospirillum lipoferum* surface saccharides

LPA lectin promoted stronger *A. lipoferum* self-agglutination than WGA (Table 1) and was therefore chosen for the experiments. Cell agglutination did not occur after treatment with sodium periodate (Fig. 1). The protein content in periodic-acid-treated cells was 525 mg litre^{-1}, 25% smaller than in untreated cells (707 mg litre^{-1}). *A. lipoferum* treated with periodic acid showed an increase in the number of dicofol–cell bonds (17·1 ng dicofol mg^{-1} protein) compared to untreated cells (8·93 ng dicofol mg^{-1} protein).

3.2 [^{14}C]Dicofol association with *Azospirillum lipoferum* proteins

Figure 2 shows the results of an electrophoresis run of total *A. lipoferum* proteins in correlation to the respective profile of [^{14}C]dicofol radioactivity of PAGE slices. From the total radioactivity bound to the cells (about 9 μg dicofol mg^{-1} protein) only 20% was recovered from the slices. Counts of radioactivity were very low, varying from 40 to 90 cpm, corresponding to 10–22 ng [^{14}C]dicofol, and did not show specificity for any protein band.

3.3 [^{14}C]Dicofol association with *Azospirillum lipoferum* lipids

The [^{14}C]dicofol bound to cells was mainly associated to lipids (80%). Applying the Folch partition method to crude lipids, we found that one-fifth of the radioactivity was provided by the hydrophilic fraction, while most of the radioactivity came from the hydrophobic part (four-fifths). From this, about 60% was found in the spots corresponding to neutral lipids, 20% was bound to phospholipids and 20% was distributed in the remaining slices (Fig. 3).

4 DISCUSSION AND CONCLUSIONS

The amount of [^{14}C]dicofol-cell bonding increases several times after photo-irradiation, as reported before.[3] The easy washing-out of this molecule from non-irradiated cells suggests that the chemical is loosely associated with the cells.

Saccharides, which are ubiquitously found in cell surfaces,[9] were not found to associate with dicofol in *A.*

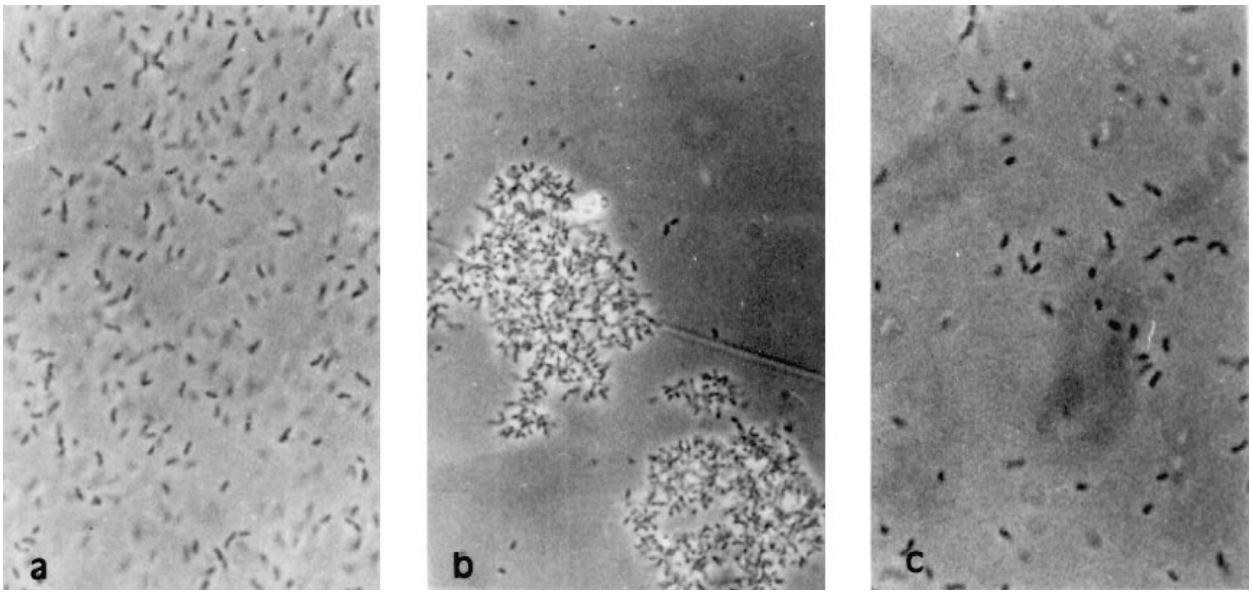

Fig. 1. Agglutination of *Azospirillum lipoferum* by lectins. 1A: control cells; 1B: after 1 h incubation with LPA; 1C: after pre-treatment with sodium periodate and subsequent incubation of 1 h with LPA.

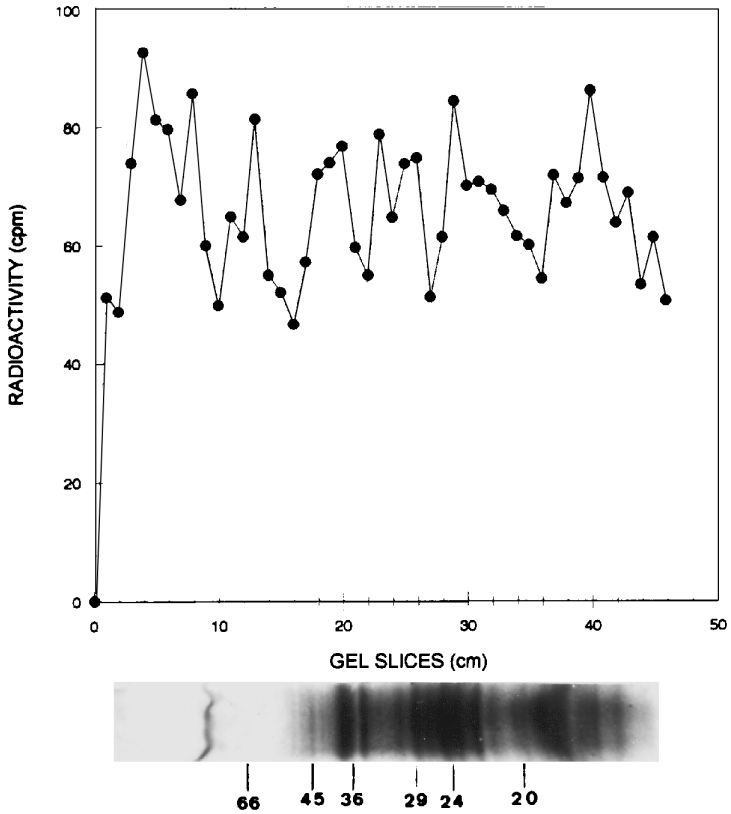

Fig. 2. Above: radioactivity measurements of [^{14}C]dicofol bound to protein in PAGE slices; below: stained PAGE of proteins bound to [^{14}C]dicofol. Molecular masses are indicated.

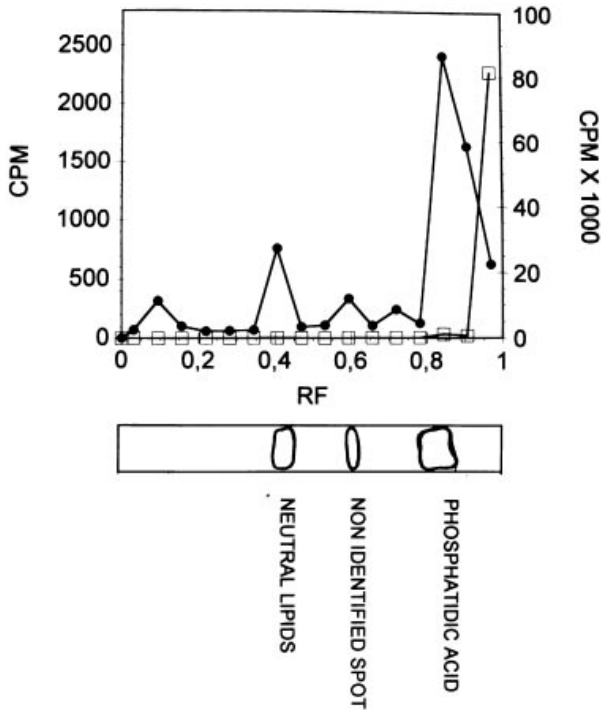

Fig. 3. Above: radioactivity measurements of TLC slices (●) lipid bound to [^{14}C]dicofol (□) [^{14}C]dicofol as control; below: distribution of lipids revealed with iodine vapour.

lipoferum. Membrane saccharides oxidation with sodium periodate enhances dicofol–cell uptake with the simultaneous loss of 25% of total bacterial proteins (Table 2). The oxidation reaction of the carbohydrates by periodic acid may have weakened the association between the cell surface carbohydrate molecules. This may be the reason why proteins have their stability decreased in the membranes and are partially removed during washing procedures. The enhancement of dicofol uptake by the cell seems to be a consequence of the reduction of membrane polarity, possibly due to protein loss, resulting in a greater membrane lipid exposition.

The distribution pattern of dicofol radioactivity after photo-irradiation in the PAGE (Fig. 2) is quite even for

TABLE 2
Photobinding of [^{14}C]Dicofol to *Azospirillum lipoferum* Treated and Not Treated with 0·1 mM Sodium Periodate

A. lipoferum	*Radioactivity* (*dpm* ml^{-1})	*Protein content* (μg ml^{-1})	*ng dicofol* mg^{-1} *protein*
Untreated	115 600	707	8·93
Treated	164 495	525	17·1

each gel slice, showing that dicofol–protein bonds are not specific for any definite protein or group of proteins. The low amount of 20% of total [^{14}C]dicofol bound to the cells is a strong argument that proteins are not the molecules directly involved in dicofol–cell association.

Experiments showed evidence for preferential association of [^{14}C]dicofol to the neutral lipids present in *A. lipoferum* cells, which are important as anchors for membrane proteins in eucariotic cells.[10] Probably dicofol accumulates more in membrane domains near proteins where neutral lipids are more abundant. The preferential association of dicofol with this class of lipid could modify membrane domains surrounding proteins. Buff and Berndt's[11] observations showed that lipophylic pesticides change such physicochemical properties as fluidity by insertion into interstitial sites in the membrane bilayer. Influence of dicofol and other organochlorines on nitrogenase in *A. lipoferum*[1,12] and on ATPase and dehydrogenase[13–15] activities was observed.

It may be supposed that the organochlorine adsorption to neutral lipids observed can change membrane microdomains that in turn influences membrane fluidity and enzymatic activities of membrane-associated proteins, constituting a general and unspecific cellular mechanism of action of organochlorine xenobiotics. Finally the incorporation of dicofol among membrane neutral lipids of *A. lipoferum* avoids the exposure of this chemical to the external aqueous medium and protects dicofol against hydrolysis, promoting its persistence.

ACKNOWLEDGEMENTS

We thank Marcos Anderson de Oliveira for his technical assistance and Profs Alane Vermelho, Arnaldo Feitosa Braga de Andrade and Allen Norton Hagler for their helpful comments.

REFERENCES

1. Langenbach, T., Mano, D. S. M., de Souza, W. & Hagler, A. N., Influence of insecticides on growth, nitrogenase activity and morphology of *Azospirillum lipoferum*. *Ci. Cult.*, **43** (1991) 207–11.
2. Walsh, P. R. & Hites, R. A., Dicofol solubility and hydrolysis in water. *Bull. Environ. Contam. Toxicol.*, **22** (1979) 305–11.
3. Mano, D. M. S., Buff, K., Clausen, E. & Langenbach, T., Bioaccumulation and enhanced persistence of the acaricide dicofol by *Azospirillum lipoferum*. *Chemosphere*, **33** (1996) 1609–19.
4. Brundl, A. & Buff, K., Photo-induced binding of 2,2′,4,4′,5,5′-hexachlorobiphenyl to cultured human cells. *Biochem. Pharmacol.*, **37** (1987) 1601–8.
5. Dobereiner, J., Mariel, I. E. & Nery, M., Ecological distribution of *Spirillum lipoferum* Beijerinck. *Can. J. Microbiol.*, **22** (1976) 1464–73.
6. Bradford, M. M., A rapid and sensitive method for the quantification of micro-organisms quantities of proteins utilising the principle of protein–dye binding. *Anal. Biochem.*, **72** (1976) 248–59.
7. Laemmli, U. K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* (*London*), **227** (1970) 680–5.
8. Folch, J., Arsove, S. & Meath, J. A., Isolation of brain strandin, a new type of large molecule tissue component. *J. Biol. Chem.*, **191** (1957) 819–31.
9. Sharon, N., *Complex Carbohydrates. Chemistry, Biosynthesis and Functions.* Addison-Wesley Publishing Company, Inc. London, 1975.
10. Olson, E. N. & James, G., The biology of lipid-containing membrane proteins. In *Dynamics and biogenesis of membranes*, ed. A. F. Jos. op dem Kamp. NATO ASI. Series H: *Cell Biology* **40** (1989) 17–27.
11. Buff, K. & Berndt, J., Interaction of DDT (1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)-ethane) with liposomal lipids. *Bioch. Bioph. Acta* (*BBA*), **643** (1980) 205–12.
12. Buff, K., Langenbach, T. & Mano, D. M. S., Effect of endosulfan on *Azospirillum lipoferum* growth, morphology, nitrogenase activity, and protein binding. *Appl. Environ. Microbiol.*, **58** (1992) 3173–6.
13. Lal, R. & Saxena, D. M., Cytological and biochemical effects of pesticides on micro-organisms. *Res. Rev.*, **73** (1980) 49–86.
14. Tripathi, A. R., The cytology and biochemistry of pesticide microbiology. *Cri. Rev. Microbiol.*, **15** (1988) 223–46.
15. Trudgil, P. W. & Widdus, R., Effects of chlorinated insecticides on metabolic process in bacteria. *Biochem. J.*, **118** (1970) 48–9.